ORIGINAL RESEARCH

NUCKS PROMOTES THE PROLIFERATION, MIGRATION AND INVASION OF LUNG CANCER CELLS THROUGH PI3K/AKT SIGNALLING PATHWAY

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ABSTRACT

Purpose: Nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS) overexpression has been reported in various types of cancers. The purpose of this study is to clarify the role of NUCKS, underlying the involvement of non-small-cell lung cancer, in the progression of lung cancer.

Methods: The small interfering ribonucleic acid (siRNA) of NUCKS was transfected into a lung cancer cell line (NCI-H460, A549, NCI-H1299 and NCI-H1975). Functional experiments (MTT assay, Annexin V-FITC/PI double staining assay, colony formation assay, wound healing assay and Transwell assay) were performed to measure the effects of NUCKS on lung cancer cell viability, migration, invasion and apoptosis.

Results: NUCKS was found to be up-regulated in lung cancer cells. Knockdown of NUCKS significantly altered lung cancer cell apoptosis, proliferation colony formation, invasion and migration. Moreover, knockdown of NUCKS attenuated the activation of the PI3K/AKT pathway in lung cancer cells.

Conclusion: NUCKS was overexpressed in lung cancer cells and played an important role in lung cancer by increasing cell growth through the PI3K/AKT signalling pathway. This *in vitro* study suggested NUCKS should be evaluated in a clinical setting as a novel biomarker and potential therapeutic target for lung cancer.

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INTRODUCTION

Lung cancer is a major causes of all cancer-related deaths worldwide and is broadly divided into small-cell lung cancer (SCLC) and non-SCLC (NSCLC) [1]. NSCLC includes adenocarcinoma and large or squamous cell carcinoma [2]. Therapeutic treatments have advanced in recent years, with an increase in the 5-year survival in patients with lung cancer from 4%–17 % over the past decade [3]. The molecular mechanism of lung cancer is complex and is primarily related to the disordering of proliferation, PI3K/AKT/mTOR signalling, oxidative stress response and cell cycle progression [4, 5]. Currently, the effective therapy for lung cancer is to block the most frequently altered pathways that relate to cell cycle, survive or metabolism. Research to identify the potential mechanisms responsible for lung cancer is crucial for the identification of novel effective targeted therapies for lung cancer.

The chromosomal protein nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS) is a

highly phosphorylated protein that is similar to the high-mobility group protein family and is involved in the tumorigenesis of various human malignancies [6, 7]. In addition, NUCKS overexpression has been reported in numerous cancers and has been shown to enhance tumor cell viability and invasion [7-9]. However, its role in the progression of lung cancer is not well understood.

Recently, some oncogenic gene variations have been identified in NSCLC [10-12]. Alterations of PIK3/AKT pathway were also reported to be involved in NSCLC cell growth [13]. The PI3K/AKT pathway is a well-known signal transduction pathway that is involved in the regulation of cell cycle processes, survival, differentiation, adhesion, motility and invasion. The activation of PI3K/AKT pathway has been reported to be related to carcinogenesis and is a critical regulator in numerous human cancers [14, 15]. In particular, AKT is a crucial mediator of carcinogenesis and the phosphorylation of AKT (p-AKT) is essential for its full activity. Furthermore, the phosphorylation of AKT on Ser473 or Thr308 is

sustain-activated in various human cancers and related to the survival rate in some cancer types, including lung cancer [16, 17]. This evidence indicates an important role of p-AKT in multiple tumor tissues, and especially in lung cancer. This study sought to answer the question of whether NUCKS is critical for the apoptosis, proliferation, invasion and migration of lung cancer cell and demonstrated that knockdown of NUCKS suppressed the growth of lung cancer cells via inactivation of PI3K/AKT pathway.

METHODS

Cell culture and transfection

The normal lung cell (BEAS-2B) and lung cancer cell lines (NCI-H460, A549, NCI-H1299 and NCI-H1975) were purchased from the American Type Culture Collection (ATCC, VA, USA). The A549 cells were maintained in F12 medium, NCI-H1299, NCI-H460 and NCI-H1975 cells were incubated in RPMI-1640 medium, and BEAS-2B cells were incubated in BEGM medium, all of them were supplemented with 10% fetal bovine serum and cultured in an incubator containing 5% CO₂. The negative control small interfering ribonucleic acid (siRNA) (si-NC) and si RNAs of NUCKS (si-NUCKS) were obtained from GenePharma (Shanghai, China). Western blot was used to determine the transfection efficiency at 48 hours after transfection.

Western blot analysis

The total protein from lung cancer cells were harvested using lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) including a protease inhibitor (Abcam, La Jolla, California, USA). A BCA protein assay kit (Sigma-Aldrich) was used to quantify the protein concentration. Thirty microgram of protein was separated by 8%–10% SDS-PAGE gel and transferred to nitrocellulose membrane (Millipore Corp., Billerica, MA, USA). The membrane was incubated with the following primary antibodies: NUCKS (1:5,000), PCNA (1:5,000), GAPDH (1:10,000), Cyclin myc (c-myc; 1:2,000), AKT (1:5,000), Bax (1:5,000), Bcl-2 (1:5,000), MMP2 (1:5,000), MMP9 (1:5,000), PI3K (1:5,000), p-AKT (1:2,000) and p-PI3K (1:2,000) overnight at 4°C. All antibodies were purchased from Abcam. Membranes were incubated with secondary antibodies for 1 hour at room temperature. Protein signals were detected using the enhanced chemiluminescence (ECL) system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Image J software (Bethesda, MD, USA).

MTT assay

The viability of cells was determined using the MTT assay (Abcam). Cells were cultured in 96-well plate, and transfected with si-NC or si-NUCKS. After incubated with MTT solution, the 96-well plates were then measured at

24, 48 and 72 hours, at a wavelength of 490 nm by microplate reader (Thermo Fisher Scientific, MA, USA).

Cell apoptosis assays

Annexin V-FITC/PI double staining assay (Thermo Fisher Scientific) was used to measure cell apoptosis. The early stage of apoptotic cells was identified by Annexin V-FITC, and the late phase of apoptotic cells was identified by PI staining. The fluorescence of cells was measured using a flow cytometer (BD Biosciences, San Jose, CA, USA). The ratio of apoptotic cells was determined by FlowJo software (Becton Dickson, San Jose, CA, USA).

Colony formation

A549 cells were cultured into 6-well plates at a density of 1×10^3 cells/well in F-12 medium containing 10% FBS. After 10 days, removed the medium, and cells were washed and stained with 1% crystal violet (Sigma) for 20 min at room temperature. The number of colonies were visualized and quantified using a light microscope.

Quantitative real-time PCR

The extraction of total RNA was performed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of mRNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The synthesized cDNA was measured by 7500 fast real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix. Data were calculated using 2^{-ΔΔCt} method. All primers used in this study were as follows:

NUCKS:

Forward: 5'-TCTGATGATGCAGATGAAGATTA-3';

Reverse: 5'-CTGCTGAGTGAGAATCATCC-3'.

β-actin:

Forward: 5'-CTTAGTTGCGTTACACCCCTTCTTG-3';

Reverse: 5'-CTGTACCTTCACCGTTCCAGTTT-3'.

Migration and invasion assays

Wound healing assay was used to determine cell migration. Cells were seeded into six-well plates at a density of 2×10^5 cells per well and transfected with si-NC or si-NUCKS and incubated for 12 hours. The sterile pipette tip was used to scrape wounds. Images for each scratch were captured at 0 and 48 hours by microscope.

Cell invasion was assayed using 8.0 μm pore filter size, and Matrigel-coated 24-well Transwell chambers (Corning Incorporated, NY, USA). After transfected with si-NC

or si-NUCKS, cells were cultured in serum-free medium and re-placed in the upper chamber and the medium contained with 10% FBS was load in the lower chamber. After 48 hours of incubation, the non-migratory cells were removed, and the invaded cells were fixed with 4 % paraformaldehyde and stained with 0.5 % crystal violet. The numbers of migratory or invasive cells were counted in three randomly selected fields.

Statistics analysis

All data were independently depicted as mean $\pm$ standard deviation (SD). Statistical analysis was analyzed using the Graphpad Prism (Graphpad Software, San Diego, CA, USA). Statistical differences among multiple groups were analyzed by one-way ANOVA and $p < 0.05$ was considered significant.

RESULTS

NUCKS overexpression in lung cancer cells.

To examine the expression level of NUCKS in lung cancer cells, qPCR was carried out in lung cancer cells (A549, NCI-H1299, NCI-H460, NCI-H1975) and normal lung cells (BEAS-2B). Compared with that in the BEAS-2B cells, NUCKS was highly expressed in lung cancer cells (NCI-H460, A549, NCI-H1299, NCI-H1975) (Figure 1A).

The results from western blot assay showed that NUCKS protein expression level was also enhanced significantly in lung cancer cells compared with that in the BEAS-2B group (Figure 1B).

To demonstrate the function of NUCKS in lung cancer cells, si-NUCKS was transfected into A549 cells, which showed the highest NUCKS expression compared with other lung cancer cell lines. The transfection efficiency

was measured by western blot assay. The data revealed that the expression of NUCKS was significantly down-regulated in si-NUCKS group compared with si-NC group (Figure 1C).

Decreased lung cancer cell proliferation by NUCKS knockdown

To determine whether NUCKS is critical for the lung cancer cell growth, the MTT and colony-forming assays were performed. The proliferation of A549 cells was markedly inhibited after transfected with si-NUCKS compared with the si-NC group (Figure 2A). Knockdown of NUCKS also led to the reduced colony formation of A549 cells compared with the si-NC group. In addition, western blot assay was used to investigate the expression of proliferation-related proteins, including c-myc and PCNA. After NUCKS knockdown, the protein expressions of c-myc and PCNA were markedly suppressed compared with the si-NC group (Figure 2C).

Increased lung cancer cell apoptosis by NUCKS knockdown

To investigate the effects of NUCKS on the apoptosis of A549 cells, the Annexin V-FITC/propidium iodide assay was performed after transfected with si-NUCKS. The number of apoptotic cells in the si-NUCKS group was increased significantly compared with the si-NC group (Figure 3A). Similarly, results from western blot assay showed that, after NUCKS knockdown, the expression of Bax was markedly increased and Bcl-2 was markedly decreased compared with the si-NC group (Figure 3B).

FIGURE 1.
NUCKS EXPRESSION LEVELS IN LUNG CANCER CELL LINES.

(A) Real-time PCR analysis of NUCKS mRNA expression in normal lung cell line BEAS-2B and lung cancer cell lines A549, NCI-H1299, NCI-H460 and NCI-H1975. (B) Western blot analysis of NUCKS protein expression in normal lung cell line BEAS-2B and lung cancer cell lines A549, NCI-H1299, NCI-H460 and NCI-H1975 (loading control: GAPDH). (C) Western blot analysis of NUCKS protein expression in A549 cell line after transfected with si-NC or si-NUCKS. Data are presented as the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus BEAS-2B or si-NC group.

FIGURE 2.
KNOCKDOWN OF NUCKS INHIBITED THE PROLIFERATION OF A549 CELLS.

(A) MTT assay of A549 cells at 0 h, 24 h, 48 h and 72 h after NUCKS expression was knocked down by siRNA transfection. (B) Colony formation assay of A549 cells after transfected with si-NC or si-NUCKS. (C) Expression levels of PCNA and c-myc were measured by western blot. Data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$ versus si-NC.

FIGURE 3.
KNOCKDOWN OF NUCKS INCREASED THE APOPTOSIS OF A549 CELLS.

(A) Annexin V-FITC assays showed that knockdown of NUCKS induced the apoptosis of A549 cells. (B) Western blot revealed that the levels of Bax were increased and Bcl-2 were decreased after knockdown of NUCKS in A549 cells. Data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$ versus control.

FIGURE 4.
INFLUENCES OF NUCKS KNOCKDOWN ON THE MIGRATION AND INVASION OF A549 CELLS.

(A) Wound healing assay of A549 cells transfected with si-NC or si-NUCKS. Representative images (left) and quantitative results (right) are shown. (B) Migration assay was performed on A549 cells transfected with si-NC or si-NUCKS. Representative images (left) and quantitative results (right) are shown. (C) Western blot revealed that expression levels of the metastasis-related proteins, MMP2 and MMP9, were decreased after knockdown of NUCKS in A549 cells. Data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$ versus control.

NUCKS knockdown inhibit lung cancer cell migration and invasion

To investigate the effects of NUCKS on A549 cell migration and invasion, the Transwell and wound healing assay were performed after transfected with si-NUCKS. After knockdown of si-NUCKS, the width of wound healing at 48 hours in the si-NUCKS group was remarkably lower than that of si-NC group (Figure 4A). Similar

results were obtained from the Transwell assay, after knockdown of NUCKS, the migratory capacity of A549 cell in si-NUCKS group was significantly attenuated (Figure 4B). Furthermore, the western blot analysis indicated that the expression levels of migration-related proteins MMP2 and MMP9 were significantly suppressed after NUCKS was knocked down in A549 cells compared to si-NC group (Figure 4C).

NUCKS knockdown attenuated the activation of PI3K/AKT signaling pathway in lung cancer cells

Since the PI3K/AKT signaling pathway plays a key role in tumorigenesis, the expression levels of key molecular in PI3K/AKT signaling pathway were investigated in A549 cells transfected with si-NUCKS. The expression levels of PI3K and AKT were found to be suppressed in A549 cells transfected with si-NUCKS as compared with si-NC group (Figure 5), suggesting that PI3K/AKT signaling pathway was regulated by NUCKS. In summary, these results indicated that knockdown of NUCKS attenuated the activation of the PI3K/AKT signaling pathway in lung cancer cells.

found to be correlated with the invasive gene signature in breast cancer [22].

In the present study, lung cancer cell proliferation, colony formation, migration and invasion in NUCKS knockdown group was significantly reduced compared with the si-NC group. Furthermore, the expression levels of cell proliferation related proteins, c-myc and PCNA, in lung cancer cells were markedly inhibited by transfection of si-NUCKS. Our results were consistent with a previous study reporting that NUCKS was associated with a high-invasive phenotype in mouse lung adenocarcinoma cell strains [6]. In addition, the present study also demonstrated that the percentage of apoptotic

FIGURE 5.
KNOCKDOWN OF NUCKS INHIBITED THE ACTIVATION OF PI3K/AKT PATHWAYS IN A549 CELLS.

Expression levels of total protein and phosphorylation of PI3K and AKT (p-PI3K and p-AKT) were measured by western blot. (loading control: GAPDH). Data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$ versus control.

DISCUSSION

This study showed that NUCKS was highly expressed in lung cancer cells (A549, NCI-H1299, NCI-H460, NCI-H1975) compared with that in normal lung cells (BEAS-2B). The altered expression of NUCKS has been reported in diverse types of human cancers, including breast cancer, colon cancer and lung cancer. NUCKS has also been reported to play essential roles in tumor growth and metastasis in almost various types of human cells. NUCKS was found to be involved in multiple biological functions by phosphorylation and its overexpression was associated with carcinoma invasion in several cancers [18, 19]. Similarly, NUCKS has been found to be highly expressed in different human cancers [6, 20].

Previous studies have indicated that the proliferative, migratory capacities of gastric cancer cells were increased by NUCKS [21]. NUCKS has also been reported to be a member of high migration protein family that is found widely in cancer cells and participates in tumor progression. A recent study has revealed that NUCKS was

cells was increased significantly and the apoptosis-related proteins, Bax and Bcl-2, were altered significantly by NUCKS knockdown. Collectively, these results suggested that the inhibition of NUCKS played a critical role in the lung cancer cell apoptosis and progression.

The effects of the PI3K/Akt pathway in cancer cell cycle process, survive, metabolism, motility and the tumor angiogenesis and inflammatory cell recruitment have been well established [23, 24]. Some studies have reported that NUCKS regulates gastric cancer cell proliferation and apoptosis through PI3K/AKT/mTOR signalling pathway [7, 21]. In the present study, the phosphorylation of PI3K and AKT were suppressed by NUCKS knockdown compared with that in si-NC group in A549 cells. These results indicated that NUCKS knockdown suppressed lung cancer cell growth through regulating PI3K/AKT/mTOR signaling pathway. The regulatory network between NUCKS and PI3K/AKT/mTOR signaling pathway needs deeper investigation.

Limitations

Although this study led to significant findings, these results are limited by several considerations. The mechanism underlying NUCKS-induced PI3K/AKT activation was not explained in the present study. Moreover, the responsible mechanisms between cell migration, invasion and NUCKS-induced PI3K/AKT activation were not fully explored. Follow-up *in vivo* and *in vitro* studies between NUCKS and PI3K/AKT/mTOR are planned.

CONCLUSION

NUCKS was overexpressed in lung cancer cells and played an important role in lung cancer by increasing cell growth through the PI3K/AKT signalling pathway. This study suggested NUCKS may be a novel biomarker and a potential therapeutic target for lung cancer.

FUNDING

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